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FACTORS AFFECTING THE STABILITY OF CRYOGENICALLY PRESERVED HUMA--ETC(U)
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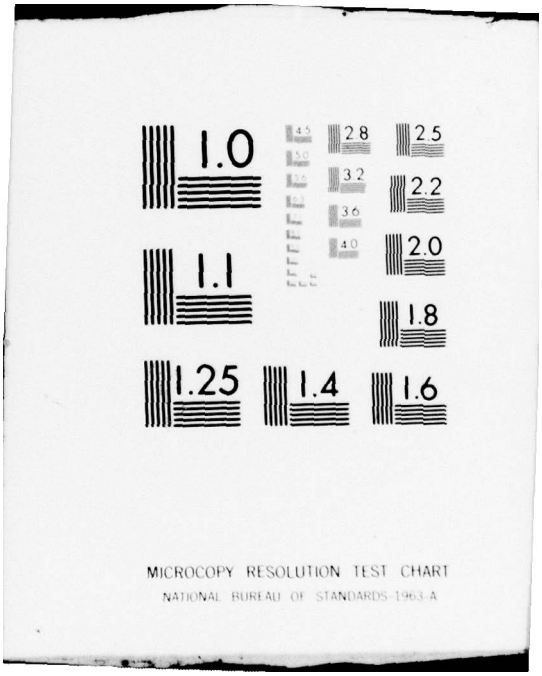
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FACTORS AFFECTING THE STABILITY OF CRYOGENICALLY PRESERVED HUMAN GRANULOCYTES

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FACTORS AFFECTING STABILITY OF CRYOGENICALLY PRESERVED HUMAN GRANULOCYTES

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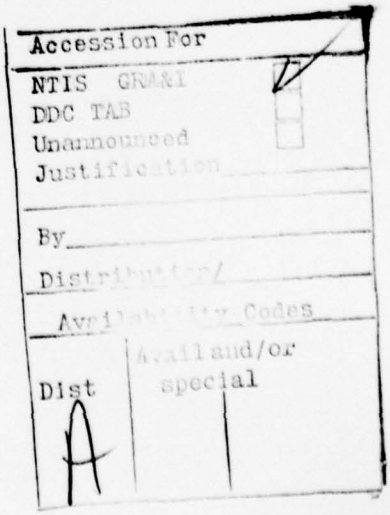

SUMMARY

Human granulocytes free of other cell types were obtained by counterflow centrifugation, cryogenically preserved, and studied for stability and function after thawing.

Isolation of granulocytes by counterflow centrifugation was optimal at reduced temperatures (4°-10°C) in phosphate buffered saline (or Ca^{++} free buffers) at pH 7.1. A stabilizing protein, or HES was required. Routinely, 1.2% human or bovine serum albumin was used. Hyperosmolar (310 mOsMoles) buffers and post isolation handling in ice water baths was optimal for cryogenic preservation. Addition of DMSO at 22° produced transient shrinkage initially which depended on the rate of addition, concentration and temperature. Within 10-15 minutes, granulocytes returned to volume, but continued to swell equilibrating for one hour at 20% larger volume. Ethidium uptake gradually increased. After 24 hours, extreme swelling, lysis and ethidium uptake was observed at the highest concentration (10%) of DMSO. DMSO induced swelling was prevented with HES.

Granulocytes (30×10^6 - 50×10^6) were frozen in 2.0 ml volumes in plastic tubes. The combination of 5% DMSO, 6% HES, 4% albumin, .026M glucose in Normosol-R at pH 7.1 produced the best yields. Granulocytes were first cooled to 4°C, then to -80°C (approx. rate 4°C per minute) in a mechanical freezer and finally stored in liquid nitrogen. Storage varied from days to months. Granulocytes were thawed at 42°C by manual twirling the freezing tubes and they were subsequently maintained in ice-water. They were diluted 3:1 dropwise with a room temperature solution of 7% HES, 1.2% albumin and

.026 M glucose in Normosol. Particle ingestion tests were conducted by incubation at room temperature for forty minutes with yeast or zymosan opsonized with autologous serum. Particles ingested were counted by microfluorimetry after two washings at 150 x g.

Granulocytes could not be cryogenically preserved in plasma or serum. Heating or prefreezing of serum was ineffective, but dialysis or addition of EDTA overcame the destructive effect of serum. Neither treatment was an improvement over the standard freeze procedure using buffered albumin and cryoprotective components. Beta-mercaptoethanol added to the freezing medium caused the production of a single homogeneous population of osmotically inert, non-viable, ethidium reactive granulocytes. This suggests that osmoregulation by granulocyte membranes is a critical requirement for cryopreservation.

Preservation efficiency is species dependent, increasing in the order of human, baboon, guinea pig, and dog. Dog granulocytes can be stored for at least 8 months in liquid nitrogen with small loss of cells and functionality.

The present efficiency of preservation of human granulocytes for 3-4 weeks of liquid nitrogen storage is 90-100% morphological and 40% functional recovery. Attempts to increase stability of thawed granulocytes with other additions to our current procedure have so far proved fruitless. These have consisted of inosine, adenine, pyruvate, gluconate, vitamin C, beta-mercaptoethanol, p-phenyl-methyl-sulfonylfluoride, and mannitol.

INTRODUCTION

Attempts to preserve white cells span the last three decades. In the 1950's, isolation methodology to obtain pure suspensions was the major effort. A variety of approaches included induced peritoneal exudates in animals, use of red cell lysins, flotation on gum acacia or serum albumin, exploitation of the differences in specific gravity between erythrocytes and leukocytes, and selective erythrocyte sedimentation using fibrinogen, dextran, and phytohemagglutinin (16). In the early 1950's, Tullis concentrated leukocytes from whole blood with dextran (14). Unlike most leukocyte preparations they were white and relatively free of red cells, and maintained at $4^{o}C$ by jelling them in 1% gelatin to prevent them from settling and clumping together. Warming the suspension to $37^{o}C$ dissoved the gelatin and resuspended the leukocytes. However, they were extremely unstable and clumped forecasting the difficulties to be experienced later in isolating and freezing granulocytes without appreciation of their interactions with other cell types, such as platelets, present in white cell concentrates (15,4).

Cryogenic preservation of leukocytes was pursued intensively in the 1960's. A hard effort by many investigators produced limited success with relatively small numbers of mixed leukocytes (1,2,3,11,13). The major limitations were technological. Only recently has the presence of lymphocytes been appreciated and the possibility of an interaction of platelets with granulocytes at low temperatures ($4^{o}C$) recognized (4). Animal studies with

cryogenically preserved granulocytes failed to demonstrate circulation in the blood (12). From the earlier preservation studies, the legacy to us in the 1970's was generally unsatisfactory methods for the isolation of granulocytes contaminated with large numbers of red cells and platelets. Cautious handling was necessary in non-wettable glass or plastic. Unavoidable and inexplicable clumping frequently happened. One principle was established; freezing required slow rates. Our first method for freezing white cells made use of these ideas (8). It employed HES as both a sedimenting and cryoprotectant (in combination with DMSO) as had previously been tried by Roy and Djerassi (12). Our contributions were the introduction of size distributions to measure cell stability and the electronic measurement of growth inhibition of E. coli by frozen-thawed granulocytes (8).

METHODS

A major advance in granulocyte preservation was made with the invention of counterflow centrifugation in the Beckman E-6 rotor. It made possible the isolation of homogeneous granulocytes from whole blood or buffy coat in a nontraumatic procedure. Studies in several laboratories show granulocytes obtained this way have properties similar to cells obtained by sedimentation (7). We have employed counterflow centrifugation to isolate homogeneous granulocytes and have used them extensively in evaluating cryogenic methods. Testing was based on size distributions for the calculation of recoveries,

microfluorescence to assess membrane stability, and particle ingestion to measure function. The yields and properties of granulocytes obtained from whole blood, buffy coats, or leukapheresis have been published (9). From small volumes of whole blood (10-20 ml) virtually 100 percent recovery of granulocytes was obtained, (40×10^6 - 80×10^6 PMN's) when the average granulocyte count is $4,000/mm^3$). These preparations consisted primarily of polymorphonuclear leukocytes with only 1-2% mononuclear cells and 3-5% red cell contamination.

Cryopreservation of granulocytes.

Three ml of cell suspension containing 1.4×10^7 cells per ml were maintained in ice water at 4^oC for 15 to 20 minutes, and then 3 ml of the cryopreservation solution chilled to 4^oC was added dropwise (1 drop per 2 seconds). The final composition of the mixture was 5% DMSO, 6% HES, 4% human serum albumin and 56 mM glucose in Normosol-R, pH 7.1. After complete mixing by swirling for 180 seconds, the contents were divided into three two ml aliquots. These were placed in 17 x 100 mm polypropylene tubes, and placed in a 4^oC ice bath. The granulocytes were maintained at 4^oC for 5 to 10 minutes and then two of the tubes were placed in a metal rack in the bottom of a Harris mechanical freezer at -80^oC where they were kept overnight, after which they were placed in liquid nitrogen at -197^oC. The third tube was used for assays of cell counts, volume distributions, and microfluorescence of prefrozen cells.

The rate of cooling achieved by storage of the polypropylene tube in a -80°C mechanical refrigerator was 4°C per minute. It was measured by placing a copper-constantan thermocouple wire in the center of the tube that was put in the -80°C mechanical refrigerator and the temperature monitored with a Honeywell Electronik III temperature recorder. In some studies controlled cooling of 1°C and 10°C per minute were carried out using a GV Planer programmed freezer R-201 that utilized liquid nitrogen to control the rates of cooling. The rates achieved by programmed freezing were also monitored with a thermocouple inserted in the center of the tube. After freezing, the tubes were stoppered and placed in liquid nitrogen at -197°C in a Linde LR-40 liquid nitrogen refrigerator. The storage period in liquid nitrogen for all tubes was 1 to 3 weeks.

Thawing and washing.

The tubes containing the frozen granulocyte suspensions were removed from liquid nitrogen and placed immediately in a water bath at 42°C, with the water above the level of frozen cells. The tubes were shaken manually while submerged with a circular motion for 120 seconds or until all but an ice pellet of 3 mm diameter remained. The tube was removed and swirling continued until the pellet was just dissolved. The tubes were cool to the touch and the temperature was 8° to 10°C. They were then placed in an ice water bath.

The thawed cells were diluted 1:4 by the dropwise addition (one drop per 2 seconds for 3 minutes) of three volumes of a solution at room temperature

composed of 7% HES, 4% human serum albumin (HSA) and 56 mM glucose in Normosol-R pH 7.1. The diluted cell suspension was then tested for stability and function as described below.

Dog granulocytes were isolated with the same techniques and media as were human cells. One hundred ml of dog whole blood anticoagulated with ACD was centrifuged in tubes and buffy coat white cells pooled and subjected to counterflow centrifugation at 315 mOsM per kg H_2O. Granulocytes were aliquoted, frozen and studied in the same way as human cells. One hundred and five ml of red cell rich white cells were collected by centrifugal leukapheresis using Volex in four passes through a pediatric (100 ml bowl) in the Model 30 Haemonetics Blood Cell Processor by Doctors Melaragno and Valeri of the Naval Blood Research Laboratory. The white cell concentrate was divided into three aliquots and each subjected to counterflow centrifugation. Of 1.7×10^9 granulocytes available in the concentrate, 1.4×10^9 (83%) were recovered. These were pooled, diluted from 13 to 20 ml with PBS albumin buffer, placed in a U-Car bag, 1000-2,100 ml capacity, and mixed with 20 ml of cryoprotective mixture in 5 increments of 4 ml added over 5 minute intervals. Excess air was removed with a syringe. The bag was placed between thin stainless steel perforated plates, clamped to form a thin section and placed flat in a styrofoam container in the bottom of a Harris mechanical freezer at -80°C. The next day the unit still between metal plates was suspended in liquid nitrogen for 8 months. Thawing was done rapidly in a 37°C bath requiring 30 seconds to rise from liquid nitrogen temperature to

2-4°C. The thawed unit was diluted with 3 volumes (120 ml) of wash solution by use of an infusion set for a drop-flow rate of about 1 drop per second with a 5 minute pause after each 40 ml. The bag was centrifuged at 350 x g for 10 minutes, and the supernatant aspirated. The pellet was resuspended with gentle mixing to 20 ml with the PBS albumin, dextrose elutriation medium.

In separate experiments dog granulocytes were isolated by counterflow centrifugation as described above but were aliquoted into plastic tubes and frozen, stored, thawed and studied identically as human cells.

The techniques for evaluating granulocytes have been published (10). Volume distributions of granulocytes obtained by counterflow centrifugation (Figure 1) showed one well defined peak corresponding to granulocytes and a small peak corresponding to red cells. No mononuclear cells were evident. In contrast mixed leukocytes obtained by sedimentation of buffy coat with HES gave three distinct populations corresponding to red cells, lymphocytes and granulocytes. The number of granulocytes within each population under investigation was readily obtained and loss of cells was determined as a decrease in the number within the distribution. Swelling caused skewing and change towards higher channel numbers while fragmentation caused a peak to form in the lowest channel numbers as the area under the main peak (proportional to the number of cells) diminished.

Microfluorescence with fluorescein diacetate (FDA) and ethidium bromide (EB) was used to assess the stability of cytoplasmic and nuclear membranes at room temperature and at 4°C while the function of granulocytes was determined

from particle ingestion measurements microfluorimetrically at room temperature. Fluorescent latex was employed in a ratio of 150 particles to 1 granulocyte in a serum (complement C-3) dependent reaction. Zymosan particles treated with ethidium bromide was also used for ingestion studies in a ratio of 200 particles per granulocyte.

MATERIALS

Phosphate buffered saline was made in 20 liter amounts by diluting isotonic phosphate buffer 1:10 with 0.14 M NaCl, making it 0.056 M with glucose, and 1.2 gm per dl with bovine albumin (Sigma) or human albumin (Hyland dialyzed). It was passed through a Falcon filter, 150 ml(#7103). The osmolality was 315 mOsMoles per kg H_2O.

Hydroxyethyl starch, CryoHES lot #P02303C, was obtained as a dry powder from the McGaw Chemical Co., Irvine, California.

PIGPA, phosphate, inosine, glucose, pyruvate, adenine was obtained from Doctor Valeri.

Para-phenylmethylsulfonyl fluoride and betamercaptoethanol was the gift of Doctor David Bing of the Center for Blood Research.

Dimethylsulfoxide, certified spectranalyzed grade was obtained from the Fisher Scientific Company.

Serum was obtained by clotting whole blood and used autologously for particle ingestion studies.

Physiogel (modified gelatine, 40 g/l in Ringer lactate solution) was obtained from the Central Laboratory of the Swiss Red Cross.

RESULTS AND DISCUSSION

Fresh granulocytes: Effects of temperature, pH, molality, divalent actions, and albumin.

Granulocytes obtained by counterflow centrifugation were investigated and determined to be similar to sedimented cells morphologically, biochemically, and functionally (9). Volume distributions showed these consist of one population of PMN's with no lymphocytes and small red cell contamination (Fig. 1). A study by Hunt and Contreras (Table) showed that granulocytes maintained at $4^{o}C$ were more stable than at $22^{o}C$ (4). A pH range of 6.9 - 7.3 was optimum, while extremes of pH (6.0) were detrimental (Figure 2). Granulocytes were considerably less stable in buffers containing divalent cations (Table). Maximum stability of cells stored at $4^{o}C$ was obtained with phosphate buffered saline (290 mOsMoles per kg H_2O at pH 7.1 - 7.2) containing 1 g per dl Physiogel or 5 g per dl of human albumin and 26 mM glucose (4). Phosphate buffered saline plus albumin and cryoprotectant components was used for subsequent cryogenic studies.

Albumin effect:

The effect of human albumin on granulocytes present during the isolation by counterflow centrifugation in the rotor, then stored at $4^{o}C$, is shown in Figure 3. Without albumin almost half of the granulocytes fragmented in 3 days. The optimum concentration was 2.0 g per dl while higher amounts (4-6 g per dl) were less effective. At 10 g per dl the density was too high

to efficiently separate granulocytes with our standard procedure in the rotor and a great many granulocytes were lost. To economize on the use of human albumin 1.2 gm per dl was used routinely.

Cryogenic preservation. Components of the system:

Previous cryogenic experience with sedimented mixed leukocytes was based on a cryoprotectant formulation containing 4% HES, and 5% DMSO in Normosol-R (Abbott Labs.), pH 7.4, an isotonic balanced salt solution (8). The cooling rate was $2^{o}C$ per minute until frozen and storage was done at $-80^{o}C$ in a Harris mechanical freezer. This overall method was adopted for elutriated granulocytes and gradually modified, adopting the principle of prefreeze and post-thaw volume control to minimize shrinking and swelling.

DMSO induced swelling. Prevention by HES:

Concentrations of DMSO required to effectively freeze-preserve granulocytes were destructive unless the temperature was quickly reduced to $4^{o}C$ or below. Thawed human cells in 5 percent DMSO at room temperature or higher exhibited unstable nuclei and fragmented rapidly. Figure 4 shows concentration dependent swelling of granulocytes by DMSO manifested by a sequential increase in volume during incubation at $4^{o}C$ and an increase in ethidium reactive cells. The initial volume, as characterized by the midrange channel number was reduced transiently on the addition of 5, 7.5 or 10% DMSO. This can be explained as osmotic shrinkage by hypertonic DMSO. However, within an hour significant swelling took place. This reached a maximum after 24 hours in 7.5% and 10% DMSO accompanied by a large increase in ethidium positive granulocytes. Whereas

good cryoprotection was observed with 10% DMSO, granulocytes were unstable on incubation at 4°C for one hour after thawing. Combination of DMSO with HES (4%) produced thawed granulocytes with greater stability on incubation at 4°C. HES alone was ineffective at these cooling rates. The combination of 5% DMSO and 4% HES reduced prefreeze swelling and ethidium reactive nuclei (Figure 5) and initially caused osmotic shrinkage. Subsequently, the PMN's swelled and reached equilibrium at a volume exceeding the untreated control. When the HES was increased to 6% the osmotic equilibrium did not exceed the initial volume or that of the control and resulted in the lowest ethidium reactive nuclei. All subsequent attemps to improve post-thawed stability were made with the 5% DMSO + HES combination. One consequence of this combination of cryoprotectants was that granulocytes were less stable after storage at -80°C for 4 months than granulocytes frozen in 10% DMSO (not shown). Accordingly after freezing to -80°C, granulocytes were stored in liquid nitrogen.

Glycerol has been reported to be an effective cryoprotectant for platelets (6) and granulocytes (17). We have not been able to substitute it for the combination of DMSO + HES in a slow freeze method. The reason we believe, is that hours are required to equilibrate glycerol with granulocytes, and removal of glycerol can not be accomplished without causing extensive swelling and cell loss. In our hands, granulocytes exposed to glycerol always became unstable, the ethidium uptake was high and they ceased to be phagocytic.

Plasma and serum effects on granulocyte freezing: Serum or plasma is essential for growth of cultured cells and helpful in preserving blood cells in the liquid state. However, as reported by Dankberg et al. (5) cryogenic preservation of granulocytes can not be effected with serum in the medium. Despite the stabilizing effect of albumin on granulocytes collected by counterflow centrifugation (described above), and its utility in granulocyte storage and cryogenics, whole serum or plasma we found to be deleterious (Figure 6). A medium of buffers, salts, nutrients and albumin was a better freeze-thaw and resuspension medium. Granulocytes frozen with serum or plasma became leaky, accumulated ethidium and lysed. The mechanism of this potentiation of freezing injury is unknown. The effect of serum on granulocyte freezing is shown in Figure 6. Increasing serum of the freezing medium from 10 to 50% produced a destructive effect on the cells. Yield, stability and function of thawed cell decreased. Neither heating at 56°C for 120 minutes to inactivate complement, nor freezing to remove Factor VIII produced a significant change. Dialysis reduced the destructive effect (Figure 7), which was probably due to the removal of divalent cations (Table 1). The addition of EDTA or EGTA to the freezing medium (Figure 8) also showed a small improvement consistent with the removal of Ca^{++}. With our standard freezing procedure, adding serum to the wash medium for the removal of DMSO was also destructive indicating the problem with serum is not relegated solely to the phase change introduced by freezing. None of the results with modified serum were improvements over the standard method without serum..

Inhibitors and nutrients: The efficiency status of our cryogenic method for cryogenic preservation of human granulocytes is greater than 90 percent morphological recovery and 40 percent functional recovery. Until thawed cells were washed and incubated with particles, the cells were stable as shown by volume and dye exclusion studies. Damage due to washing may well reflect damage to membranes inflicted during freezing and thawing. An instensive effort to minimize this damage with osmotic control of volume before and after freezing has helped us get to our present level of efficiency. A variety of media stabilizers, enzyme and free radical inhibitors and substrates have been introduced into media for granulocytes in an attempt to improve post-thawed stability. One goal was to inhibit phagocytosis as a potentially cell destructive event by preventing lysosome and granular discharge of phagocytic enzymes and bacteriocidal radicals. Another approach was to support the metabolism of granulocytes optimally by the addition of substrates. We have tried a group of these as additives to freezing solutions. Buffers containing divalent cations, such as Hanks' Wallace, or serum were unsuitable for freezing granulocytes. The best medium in our hands was Normosol-R a balanced salt buffer containing gluconate. Gluconate is a reducing substance and an oxidation product of glucose with potential metabolic significance. The small improvement in stability of thawed granulocytes with Normosol-R cannot be attributed to gluconate since we could demonstrate no effect of it alone. Vitamin C did not effect freeze-thaw stability, while β-mercapthoethanol was destructive and potentiated freezing injury

(Figure 9). PIGPA, solution used by Valeri and associates containing phosphate inosine glucose pyruvate, and adenine to support the metabolism of red cells and elevate 2,3-DPG was without effect. Likewise, the protease inhibitor, para-phenylmethylsulfonyl fluoride was destructive to granulocytes when frozen and thawed.

Cells frozen with β-mercaptoethanol in the medium differed significantly in several respects from those frozen without it (Figure 9). The thawed cells consisted of a homogeneous small population. All were ethidium reactive, indicative of leaky nuclear membranes. Further treatment, such as dilution to reduce DMSO or incubation did not change the population. In contrast, without β-mercaptoethanol, thawed cells were much larger and relatively unreactive with ethidium. Dilution to reduce DMSO caused two populations to form, one which was osmotically active as evident from swelling. Ethidium reactivity increased to 28% in rough proportion to the number of small cells. On incubation for two hours, the populations shifted so that nearly 75% were small (#50), and 79% were ethidium positive. The data suggests that membrane damage due to freezing or β-mercaptoethanol renders granulocytes increasingly solute and water permeable, resulting in a population of small prelytic granulocytes which are non-viable.

Cryopreserved granulocytes of lower animals are far superior in quality to human granulocytes. Preservation efficiency is species dependent, increasing in the order, human, baboon, guinea pig, and dog. Dog granulocytes can be stored in liquid nitrogen for a least 8 months with small loss of cells and functionality. The results to two experiments with elutriated dog

granulocytes frozen in tubes and stored in liquid nitrogen for 2, 3, and 9 days is shown in Figure 10. Granulocytes were also obtained by counterflow centrifugation after leukapheresis, frozen in U-Car platelet bags, and stored in liquid nitrogen for 8 months. Of those frozen in tubes, 97% were recovered after thawing and showed good stability and phagocytic indices. Latex ingestion was: high 16%, medium 52%, low 26%, and zero 6%. Yeast ingestion was: high 15%, low 54%, and zero 31%. Fluorescein positive cells were 80% and ethidium positive cells were 20%. The data for the granulocytes obtained by leukapheresis and counterflow centrifugation and frozen in bags demonstrated nearly equal stability after eight months in liquid nitrogen. With the exception of human monocytes, this is the best data we have obtained with human blood phagocytes.

The biggest problem yet to solve is the instability of granulocytes after thawing. When maintained at $4^{o}C$, they were stable for hours. However, centrifugation, elevation of temperature to 20^{o} or $37^{o}C$, or washing caused extreme damage and clumping. The means to reduce this trauma is the basis for current investigation.

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LEGENDS

Figure 1. Volume distributions of buffy coat sedimented with HES (8) (upper) and elutriated counterflow centrifuged granulocytes: The numbers over each peak are midrange (medians) corresponding to red cells (#10), lymphocytes (#33), and granulocytes (#58, #56).

Figure 2. pH effect on granulocyte volume. Granulocytes were incubated at $4^{o}C$ for 90 hours at pH 6.0 (upper curve) and pH 7.2 (lower curve). The numbers under the curves are the numbers of granulocytes x 10^{3} in 0.1 ml of the suspensions. The numbers over the curves are median channel numbers.

Figure 3. Albumin effect on granulocytes during counterflow centrifugation. Granulocytes were isolated in PBS containing 0, 1.2, 2, 4, 6, and 10% human albumin (Hyland, dialyzed). The hatched bars give percent of cells recovered (REC) and the solid bars give the percent ethidium positive cells (EB). The numbers over the bars are median channel numbers of the volume distributions.

Figure 4. DMSO induced swelling of granulocytes at $4^{o}C$. The median channel numbers (top) and percent ethidium positive granulocytes (bottom) are plotted for 5% (diagonal bars), 7.5% (black bars) and 10% (cross hatched bars) DMSO at $4^{o}C$ for 24 hours. The open bars marked C represent controls without DMSO.

Figure 5. Correction of DMSO induced swelling by HES. Granulocyte volume represented as median channel numbers is shown in the upper curves. The percentage of ethidium positive granulocytes is given in the bars at the bottom.

Figure 6. Serum effect on granulocyte cryopreservation. The diagonal stripes give the percentage of granulocytes recovered for each treatment and the solid bars give percentage of ethidium positive cells. PF is prefrozen, PFI is prefrozen and incubated at 37°C for 30 minutes, PT is post-thawed, PTD is post-thawed and diluted, and PTWI is post-thawed and incubated at room temperature for one hour and washed once by centrifugation. The numbers over the bars are median channel numbers. PTW is thawed and washed. STD is the average and standard deviation of seventeen standard freezings.

Figure 7. Effect of dialyzed serum on granulocytes cryopreservation. The chart is organized similarly to Figure 6.

Figure 8. Effects of EDTA and EGTA on granulocyte cryopreservation. The chart is organized similarly to Figure 6.

Figure 9. β-Mercaptoethanol effect on cryopreserved granulocytes. The curves on the left represent granulocytes frozen with the standard method, those on the right similarly with 0.7 mM β-mercaptoethanol. PTD means thawed and diluted slowly to 1% DMSO. PTDI represents an incubation at room temperature after thawing and diluting the granulocytes. The numbers within the curves represent the cells remaining after each treatment while those above are medians. The numbers under G mean the percentages of fluorescein producing cells after each treatment and the numbers under R mean the percentages of ethidium positive cells.

Figure 10. Cryopreserved dog cells. Dog granulocytes obtained by elutriation were frozen in tubes and thawed after 3.5, 5, and 9 days in liquid nitrogen. The bottom bars depict an experiment with dog granulocytes obtained by leukapheresis and counter-flow centrifugation, frozen in U-Car platelet bags, and stored in liquid nitrogen for 8 months. The bars mean the same as in the other figures. Phagocytosis is a composite bar shown for latex (L) and yeast (Y) in the upper left for the control, and in the extreme right bars for each time period. The bottom (horizontal bars) is high capacity, >15 latex particles per cell, or >5 yeasts per cell. The middle (dotted) region represents 5-15 particles per cell or 1-5 yeasts per cell while the top (latex only) (slant) bar represents 1-5 latex particles per cell.

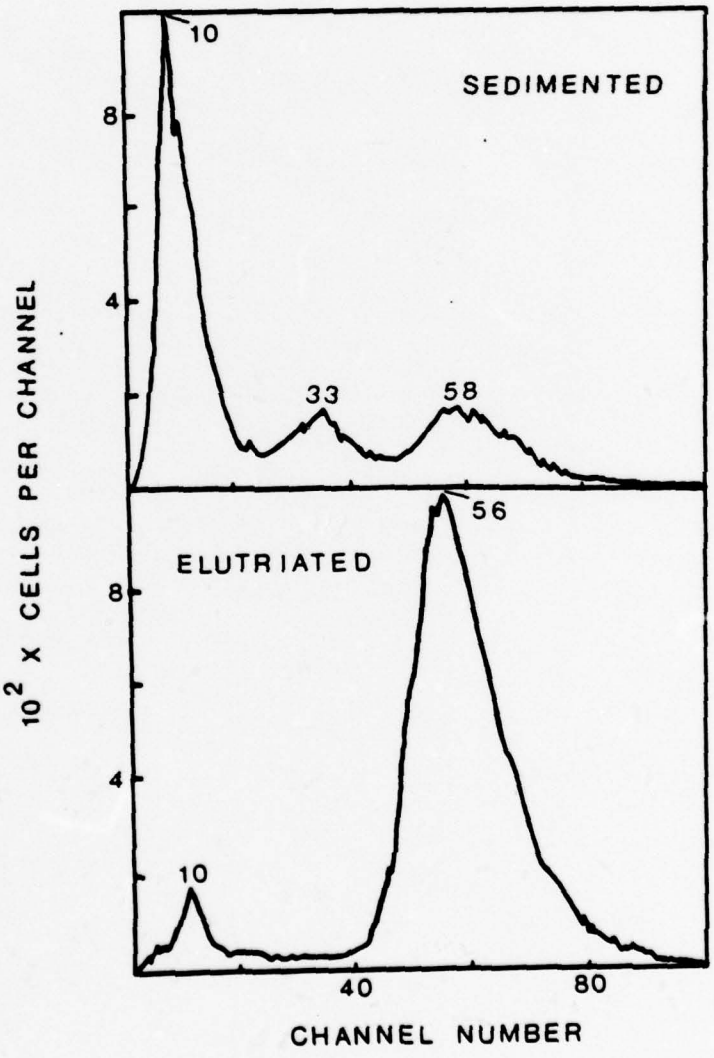
10
SEDIMENTED
8
4
33
58
56
ELUTRIATED
8
4
10
40
80
CHANNEL NUMBER
10^2 X CELLS PER CHANNEL

FIG. 1

TABLE

Temperature and divalent cation effects on stored granulocytes

Treatment	Days stored	Number of cells in volume distribution (x 10^3)	Median channel #
4^oC	0	14.0	60
PBS + Albumin	1	13.6	62
	2	13.1	67
22^oC	0	14.0	60
	1	8.9	74
	2	1.0	80
HBSS	0	6.5	64
(-Ca-Mg)	1	5.4	67
	2	5.5	68
4^oC	3	4.7	65
HBSS	0	6.3	67
(+Ca+Mg)	1	2.9	72
	2	2.3	67
	3	2.0	60

Granulocytes stored at 4^oC and 22^oC in PBS + albumin and dextrose as described in Methods.
HBSS is Hanks' balanced salt buffer with or without Ca^{++} and Mg^{++}. They were incubated as described (4). The number of cells in the the volume distribution are contained in 0.1 ml sampled by the Channelyzer of the 40 ml sterile incubation containing 2.5×10^5 - 7.0×10^5 cells.

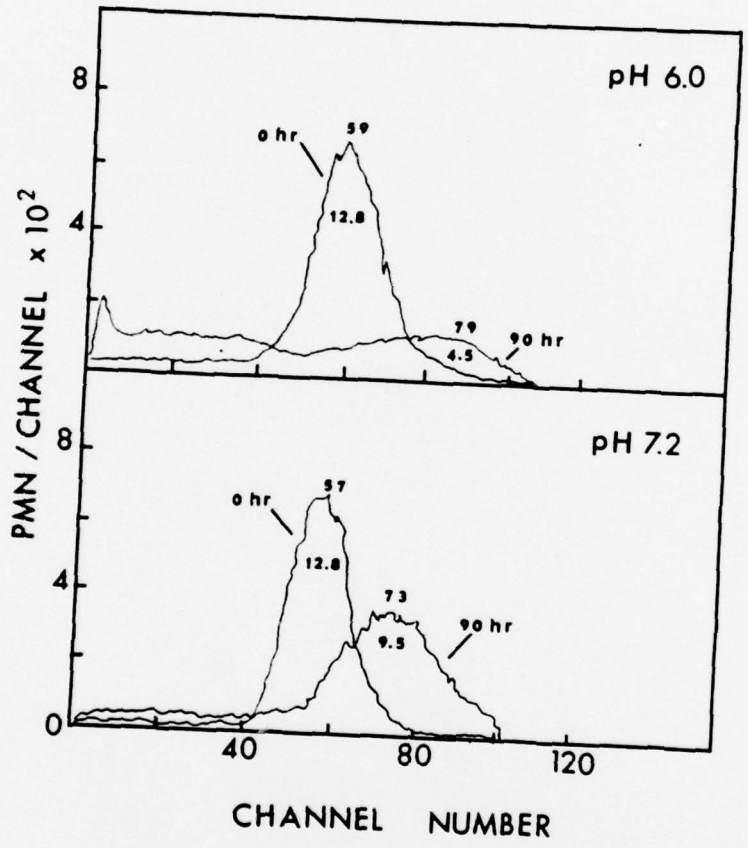
pH 6.0
0 hr
59
12.8
7.9
4.5
90 hr
pH 7.2
0 hr
57
12.8
7.3
9.5
90 hr
8
4
0
40
80
120
PMN / CHANNEL x 10²
CHANNEL NUMBER

FIG. 2.

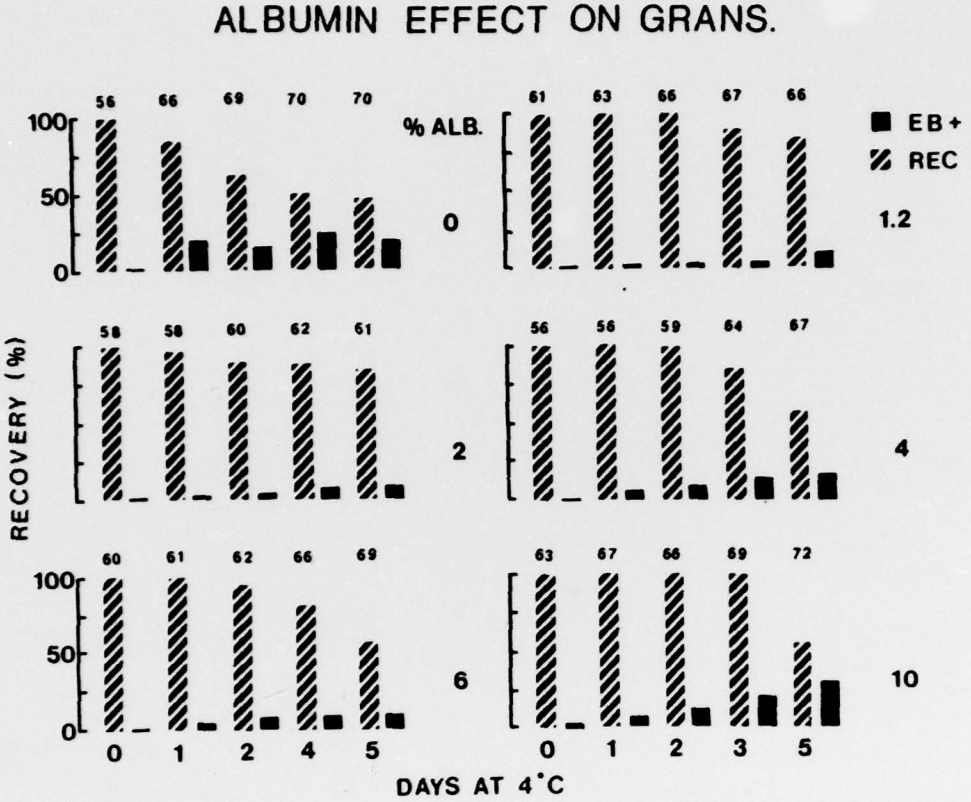
ALBUMIN EFFECT ON GRANS.
RECOVERY (%)
100
50
0
% ALB.
EB+
REC
0
1.2
2
4
6
10
56 66 69 70 70
61 63 66 67 66
58 58 60 62 61
56 56 59 64 67
60 61 62 66 69
63 67 66 69 72
0 1 2 4 5
0 1 2 3 5
DAYS AT 4°C

FIG. 3

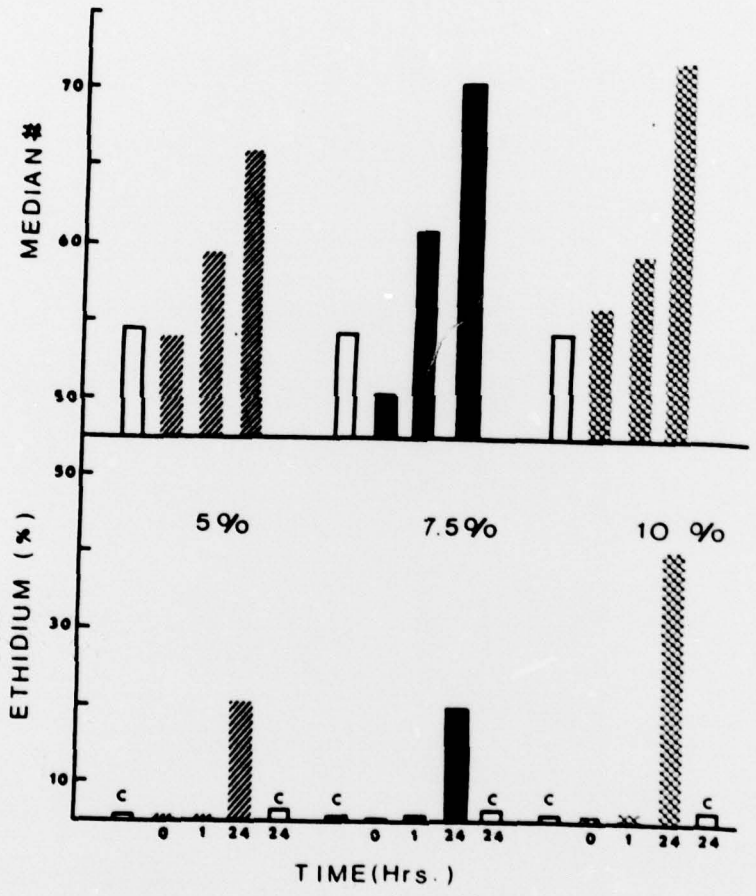

FIG. 4

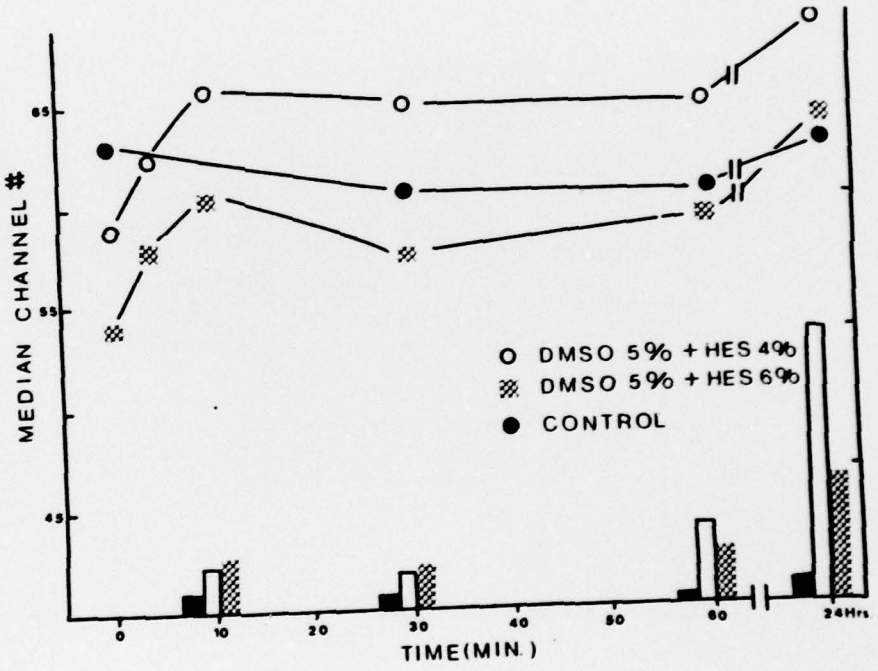
MEDIAN CHANNEL #
65
55
45
O DMSO 5% + HES 4%
DMSO 5% + HES 6%
CONTROL
0
10
20
30
40
50
60
24Hrs
TIME(MIN.)

FIG. 5

SERUM EFFECT ON GRANS.

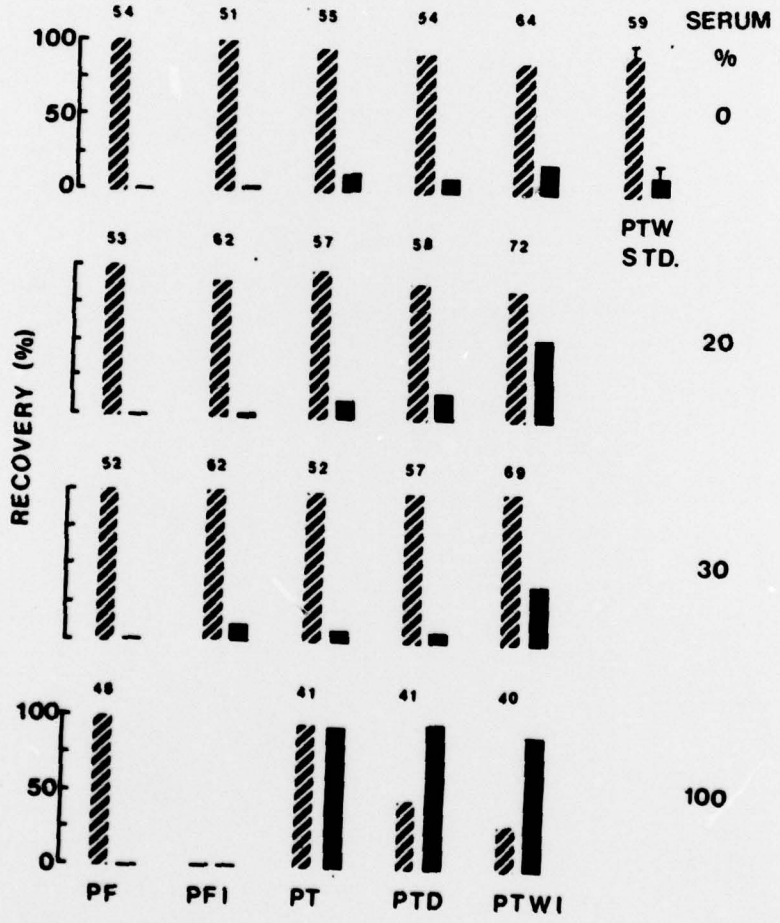

FIG. 6

EFFECT OF DIALYZED SERUM ON GRANS.

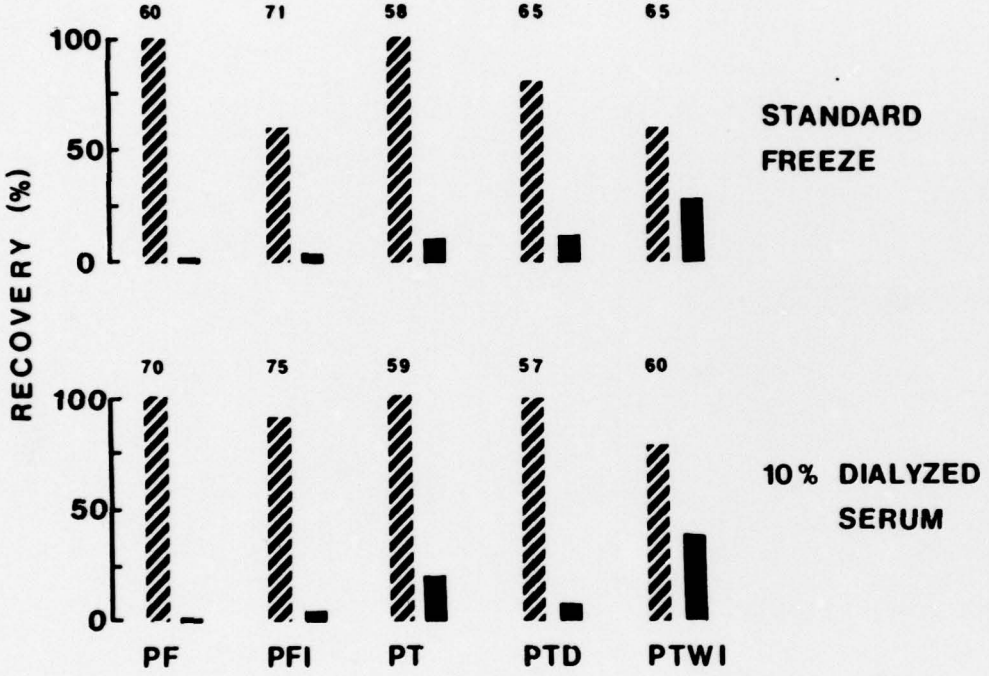

FIG. 7

EFFECTS OF EDTA AND EGTA ON GRANS.

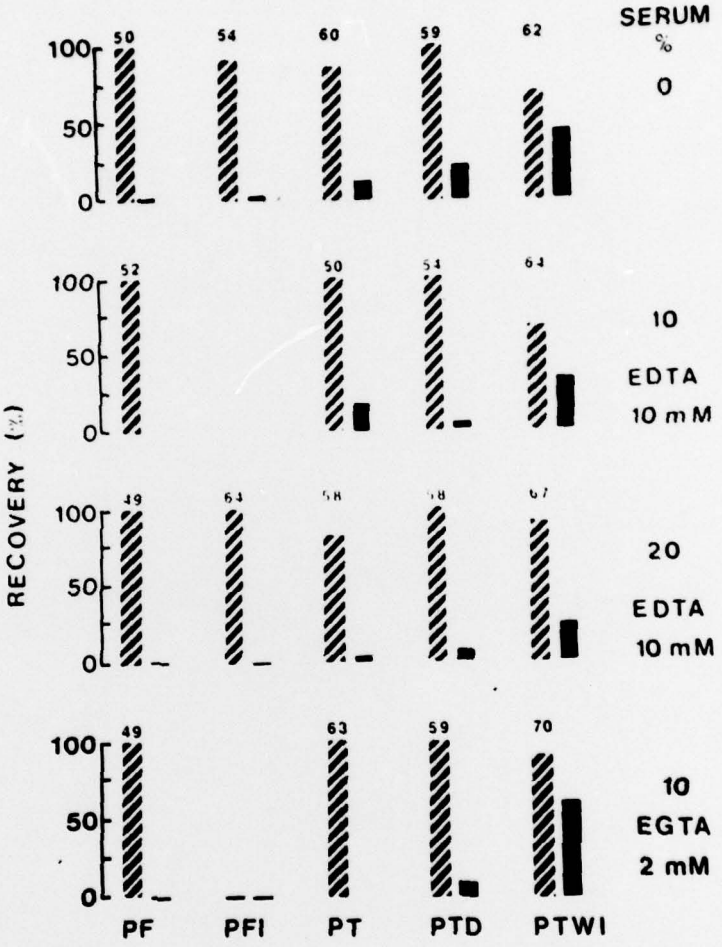

FIG. 8

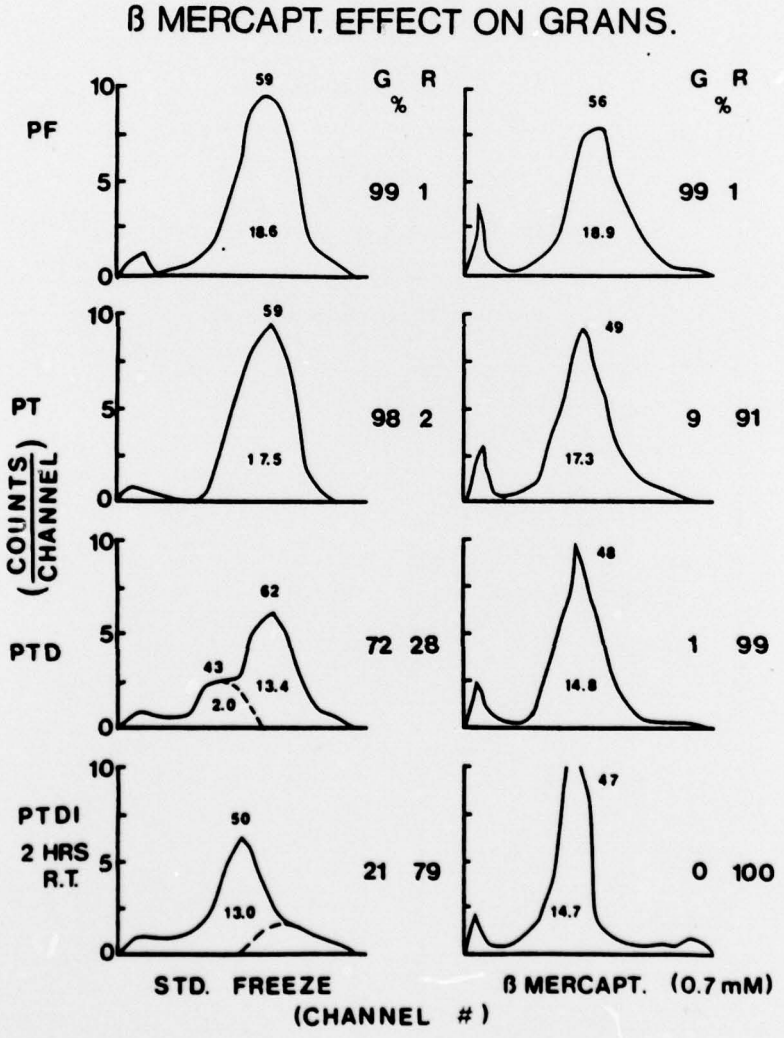
ß MERCAPT. EFFECT ON GRANS.
PF
PT
PTD
PTDI
2 HRS
R.T.
(COUNTS/CHANNEL)
G %
R %
99 1
98 2
72 28
21 79
99 1
9 91
1 99
0 100
59
18.6
59
17.5
62
43
2.0
13.4
50
13.0
56
18.9
49
17.3
48
14.8
47
14.7
STD. FREEZE
ß MERCAPT. (0.7 mM)
(CHANNEL #)

FIG. 9

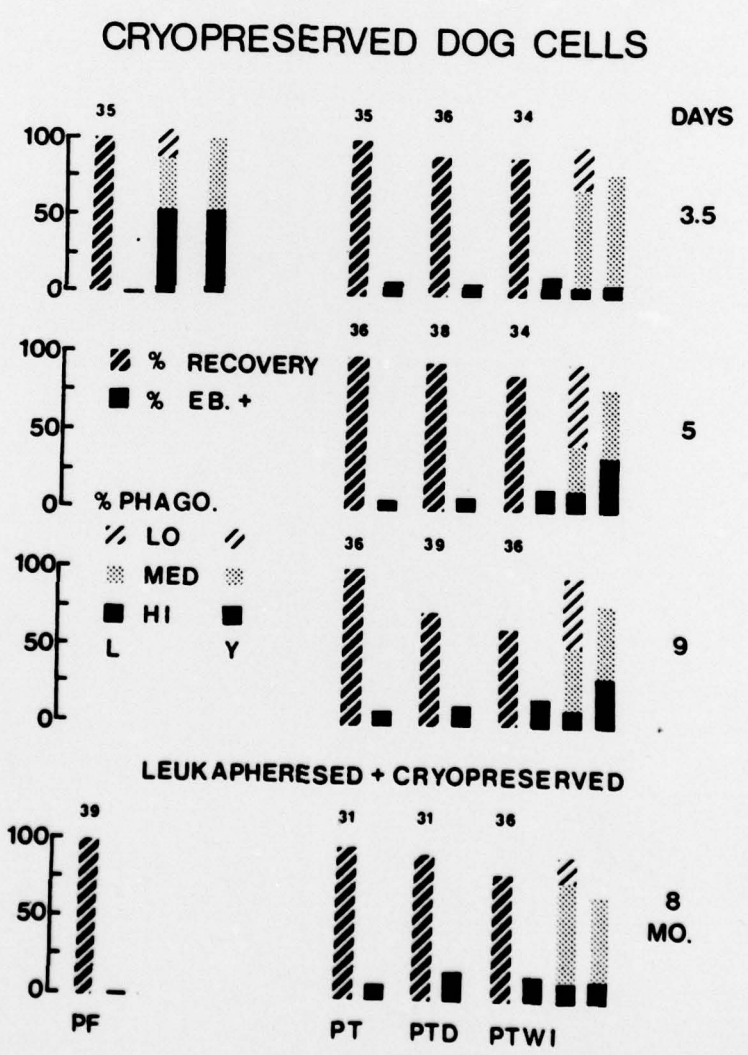
CRYOPRESERVED DOG CELLS
DAYS
3.5
5
9
% RECOVERY
% EB. +
% PHAGO.
LO
MED
HI
L
Y
LEUKAPHERESED + CRYOPRESERVED
8
MO.
PF
PT
PTD
PTWI

FIG. 10

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

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granulocytes, counterflow centrifugation, volume distributions, phagocytosis, freeze preservation, microfluorimetry.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Human granulocytes free of other cell types were obtained by counterflow centrifugation, cryogenically preserved, and studied for stability and function after thawing.

Isolation of granulocytes by counterflow centrifugation was optimal at reduced temperatures (4°-10°C) in phosphate buffered saline (or Ca^{++} free buffers) at pH 7.1. A stabilizing protein, or HES was required. Routinely, 1.2% human or bovine serum albumin was used. Hyperosmolar (310 mOsMoles)

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buffers and post isolation handling in ice water baths was optimal for cryogenic preservation. Addition of DMSO at 22° produced transient shrinkage initially which depended on the rate of addition, concentration and temperature. Within 10-15 minutes, granulocytes returned to volume, but continued to swell equilibrating for one hour at 20% larger volume. Ethidium uptake gradually increased. After 24 hours, extreme swelling, lysis and ethidium uptake was observed at the highest concentration (10%) of DMSO. DMSO induced swelling was prevented with HES.

Granulocytes (30×10^6 - 50×10^6) were frozen in 2.0 ml volumes in plastic tubes. The combination of 5% DMSO, 6% HES, 4% albumin, .026M glucose in Normosol-R at pH 7.1 produced the best yields. Granulocytes were first cooled to 4°C, then to -80°C (approx. rate 4°C per minute) in a mechanical freezer and finally stored in liquid nitrogen. Storage varied from days to months. Granulocytes were thawed at 42°C by manual twirling the freezing tubes and they were subsequently maintained in ice-water. They were diluted 3:1 dropwise with a room temperature solution of 7% HES, 1.2% albumin and .026 M glucose in Normosol. Particle ingestion tests were conducted by incubation at room temperature for forty minutes with yeast or zymosan opsonized with autologous serum. Particles ingested were counted by microfluorimetry after two washings at 150 x g.

Granulocytes could not be cryogenically preserved in plasma or serum. Heating or prefreezing of serum was ineffective, but dialysis or addition of EDTA overcame the destructive effect of serum. Neither treatment was an improvement over the standard freeze procedure using buffered albumin and cryoprotective components. β-mercaptoethanol added to the freezing medium caused the production of a single homogeneous population of osmotically inert, nonviable, ethidium reactive granulocytes. This suggests that osmoregulation by granulocyte membranes is a critical requirement for cryopreservation.

Preservation efficiency is species dependent, increasing in the order of human, baboon, guinea pig, and dog. Dog granulocytes can be stored for at least 8 months in liquid nitrogen with small loss of cells and functionality.

The present efficiency of preservation of human granulocytes for 3-4 weeks of liquid nitrogen storage is 90-100% morphological and 40% functional recovery. Attempts to increase stability of thawed granulocytes with other additions to our current procedure have to far proved fruitless. These have consisted of inosine, adenine, pyruvate, gluconate, vitamin C, β-mercaptoethanol, p-phenyl-methyl-sulfonylfluoride, and mannitol.

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